American Fern Journal 79(4): 136–143 (1989)

The Effects of Temperature and Selected Growth Regulating Substances on Sporulation in the Aquatic Fern *Azolla*

Y. R. Herd and Elizabeth G. Cutter
Plant Science and Cytogenetics Group, Department of Cell and Structural Biology, Williamson Building, The University, Manchester, M13 9PL, U.K.

I. Watanabe
International Rice Research Institute, Manila, Philippines

Further exploitation of the use of *Azolla* in rice cultivation has been restricted by its infrequent and unreliable sexual reproductive cycle. Production of new strains and hybrids of *Azolla* species is dependent on the ability to induce sporocarps in culture at will. Although the effects of temperature on the vegetative growth of different *Azolla* species have been the subject of many previous publications (Holst & Yopp, 1979; Peters et al., 1980; Talley & Rains, 1980; Tung & Watanabe, 1983; Watanabe & Berja, 1983), few workers have considered the possibility that temperature changes may induce sporulation (Ashton, 1977; Watanabe et al., 1981). The effects of some growth regulating compounds on the vegetative growth of *Azolla* have also been reported (Nickell, 1961). The aim of the present work was to subject a readily available number of different species and strains of laboratory-cultured *Azolla* to a range of temperature regimes, and to culture solutions modified by the addition of growth regulating substances, in an attempt to induce sporulation.

Materials and Methods

Stocks of *Azolla* were maintained in a Fisons Fitotron 600H growth cabinet in the University of Manchester after their collection from the International Rice Research Institute in the Philippines. Plants were cultured in 250 ml beakers containing 150 ml of a nitrogen-free nutrient solution as described previously (Watanabe et al., 1977) and were given a 12 hour photoperiod with a light intensity of 30 kilo lux (80 watts)/m^2 at frond level. The light source was warm-white fluorescent tubes with four 40 W tungsten bulbs. Loss of medium due to evaporation was made good with autoclaved deionized water, and the solution was changed every four weeks. Day temperature was 26°C while night temperature was set at 18°C, both with 70% relative humidity. The temperature of the medium was checked and found to be approximately the same.

The *Azolla* collection comprised eleven strains of *A. pinnata*, numbers 2, 5, 9, 13, 17, 22, 32, 35, 40, 701, and 704 of the IRRI culture collection; one strain of *A. nilotica* (501); three strains of *A. filiculoides* (101, 105, 108); four strains of *A. mexicana* (201, 202, 203, and 204); two strains of *A. caroliniana* (301 and 302); and three strains of *A. microphylla* (406, 417, and 418). Identification of some of the IRRI strains is still tentative, but 201 seems likely to be *A. mexicana*. In addition to the IRRI strains, one extra strain of *A. pinnata* from Manikganj in

Bangladesh (courtesy of D. Livingstone, Department of Botany, University of Durham), labelled *A. pinnata* (Man.), was also cultured.

Experiments to determine the effects of temperature upon sporulation were set up in a second growth cabinet with duplicate cultures. Both day and night temperatures were lowered by 5°C in the first experiment; other conditions remained the same. This experiment was run over about six months. In a second experiment of the same duration the night temperature only was reduced by 5°C. Cultures were examined for sporulation every 4 weeks under a dissecting microscope. Each culture comprised numerous plants, some 65% of which were sampled on each occasion.

Four growth substances, abscisic acid (ABA), triiodobenzoic acid (TIBA), gibberellic acid (GA_3), and ethrel were individually tested at concentrations of 1, 10, and 100 mg/liter of nutrient solution on two strains of *A. pinnata* (5 and *A. pinnata* Man.) and two strains of *A. filiculoides* (105 and 108). The other growth conditions were the same as those in the control (stock) cabinet. Each experiment was run over a period of three months.

Mature megasporocarps from the control and lower temperature cultures were prepared for scanning electron microscopy by freeze-drying. Following the removal of the indusium, the megaspore apparatus was attached to a stub with double-sided tape and sputter-coated with gold. The stubs were then examined in a Cambridge S-150 scanning electron microscope.

Results

Table 1 shows the effects of various temperature regimes on the sporulation of different strains of *Azolla*. A culture was positively (+) identified as sporulating if a substantial part of the culture was covered in sporocarps, regardless of whether they were mostly megasporocarps or microsporocarps or a combination of both types. If only one or two sporocarps were produced on up to ca. 6 plants a note was made of this as −(+), but the culture was generally regarded as not sporulating.

Using the stock cabinet as a control for comparison with the temperature experiments, sporulation in three strains of *A. pinnata* (5, 704, and *A. pinnata* Man.) and in three strains of putative *A. mexicana* (202, 203, 204), was induced by an overall reduction of both the day and night temperatures of 5°C.

Sporulation in all strains of *A. microphylla* was maintained at these lower temperatures and in *A. mexicana* (201) sporulation was inhibited. However, it was found that mostly microsporocarps were formed in the three strains of *A. pinnata*, and that when megasporocarps were formed they were often (but not always) abnormal. The scanning electron micrographs in Figure 1 show examples of the normal and abnormal megaspore apparatus of *A. pinnata* and of a normal massula formed under the same conditions. The formation of supernumerary floats seems to be a common abnormality in this material, although deformed megaspores (Fig. 1c) are often found in these cultures. Scanning electron microscopy of the three strains of *A. mexicana* failed to show any normal megasporocarps although massulae within the microsporangia usually appeared to be fully formed (Fig. 2). The megaspore apparatus of the

TABLE 1. Effect of Temperature on Sporulation.

Species	Strain	Temperature(°C)		
		Day/Night 26/18 (Control)	Day/Night 21/13	Day/Night 26/13
A. pinnata	2	−[1]	−	−
	5	−	+[2]	+
	9	−(+)[3]	−	−
	13	−	−	−
	17	−	−	−
	22	−	−	−
	32	−	−	−
	35	−	−	−
	40	−	−	−
	701	−	?[4]	+
	704	−	+	+
	Man.	−(+)	+	+
A. nilotica	501	+	?	?
A. filiculoides	101	−	−	−
	105	−(+)	−	−
	108	−(+)	−	−
A. mexicana	201	+	−	+
	202	−	+	−
	203	−(+)	+	−
	204	−(+)	+	−(+)
A. caroliniana	301	−	−	−
	302	−	−	−
A. microphylla	406	+	+	−(+)
	417	+	+	−(+)
	418	+	+	+

−[1] Denotes absence of sporocarps in a culture.
+[2] Denotes culture covered in sporocarps.
−(+)[3] Denotes presence of one or two sporocarps on a few plants in a culture.
?[4] Denotes death of plants in a culture during the experiment.

three strains showed varying degrees of abnormality when compared with the normal megaspore apparatus of *A. mexicana* 201 (Fig. 3) which was produced in culture in the control cabinet. The "abnormal" megaspore apparatus described here did not resemble the normal structures of other species of *Azolla* either.

A reduction by 5°C of the night temperature only, induced sporulation in four strains of *A. pinnata* (5, 701, 704, and *A. pinnata* Man.) in comparison with the controls. These conditions appeared to reduce or inhibit sporulation in strains 406 and 417 of *A. microphylla*.

The results of the growth regulating substance experiments were disappointing in that none of the compounds tested at the concentrations used promoted sporulation. In fact higher concentrations of ethrel (100 mg/l) and TIBA (10 mg and 100 mg/l) resulted in death of the cultures.

DISCUSSION

The results obtained in the temperature experiments confirm previous suggestions (Ashton, 1977; Watanabe et al., 1981) that temperature influences

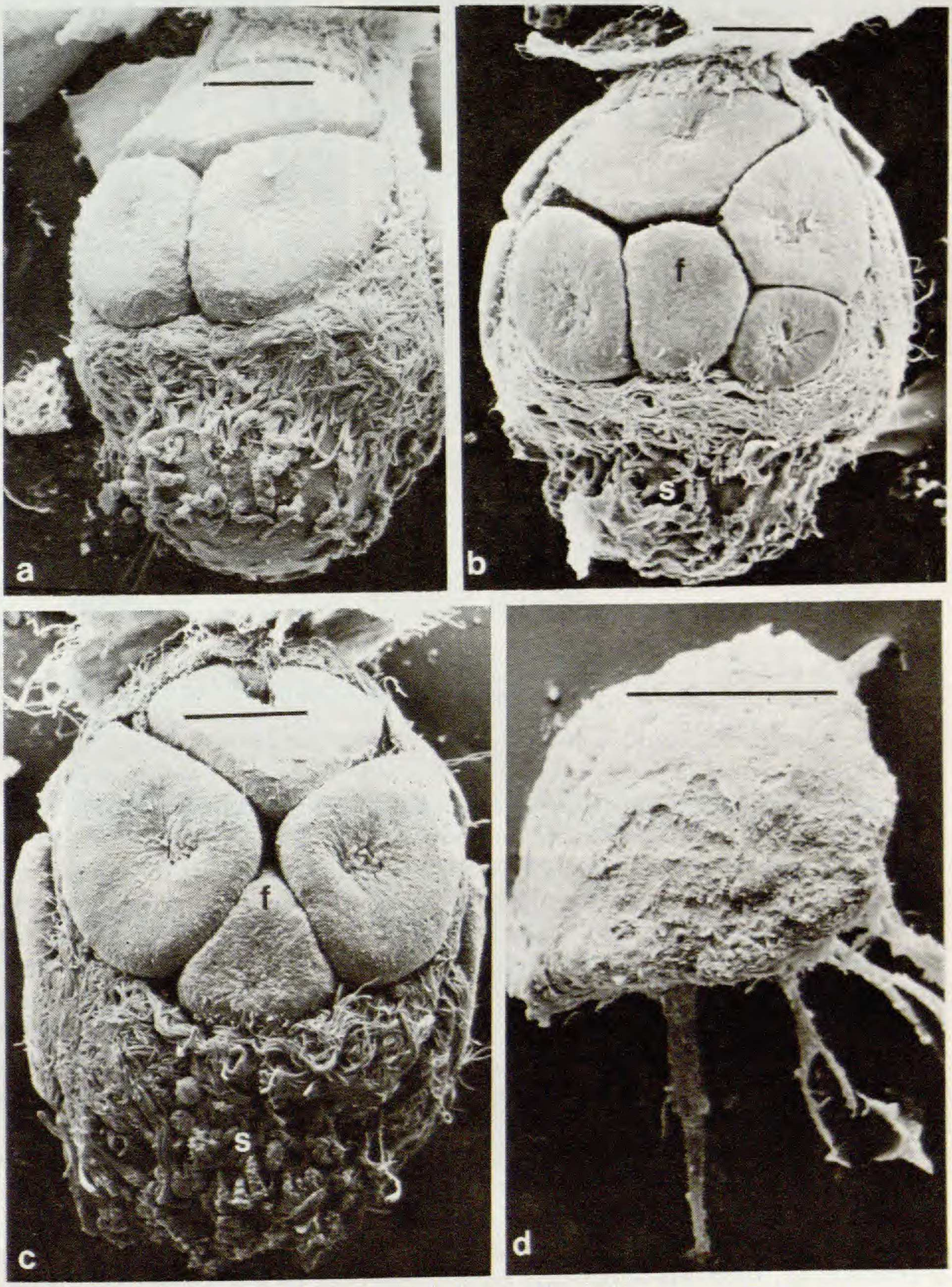

FIG. 1. Megaspore apparatus and massula of *Azolla pinnata* strain 5. a, Normal megaspore apparatus from a culture in day/night temperatures of 21/13°C. b, Abnormal megaspore apparatus with multiple float (f) formation above a normal megaspore (s) from a culture in day/night temperatures of 21/13°C. c, Abnormal megaspore apparatus with a supernumerary float (f) over a deformed spore (s). Culture conditions as above. d, Normal massula produced in culture in the same conditions as above. All bars = 100 μm.

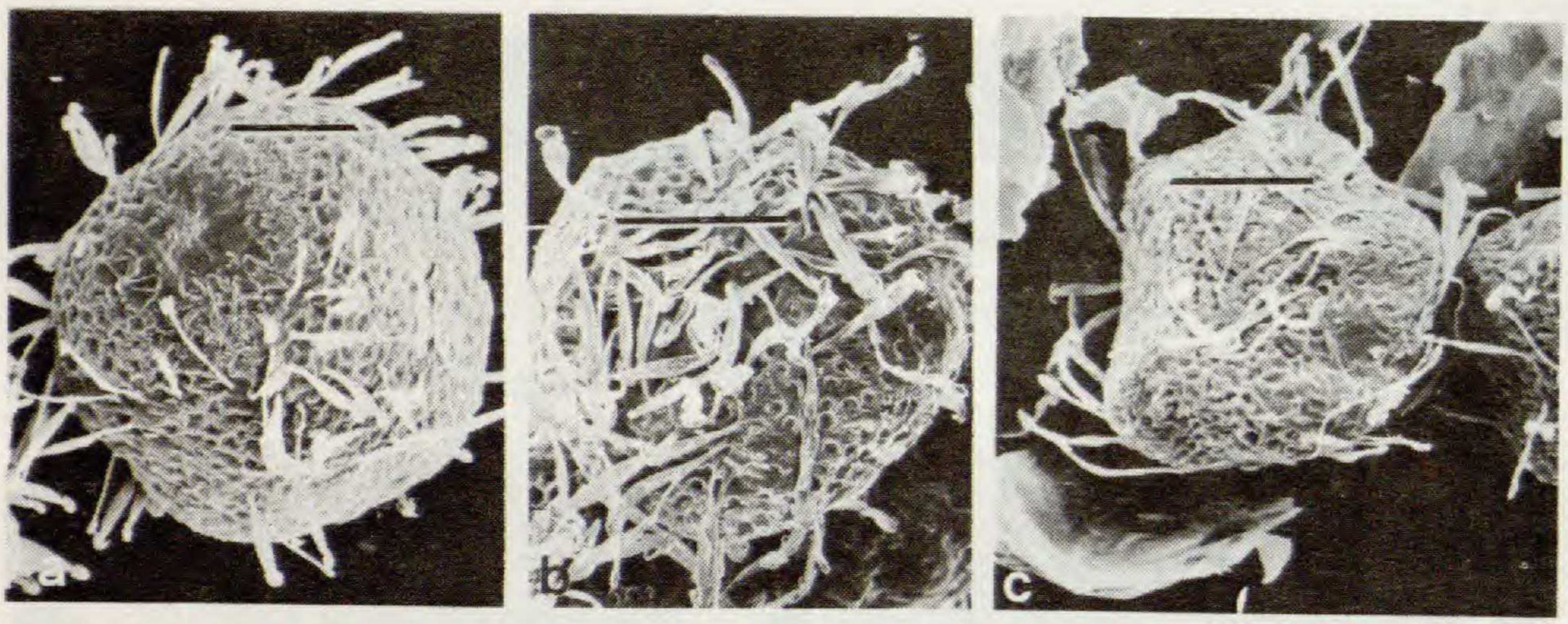

FIG. 2. Megaspore apparatus and massula of *Azolla mexicana* cultured under reduced temperature conditions (21/13°C). a, Megaspore apparatus and massula of strain 202. b, Normal massula of strain 203. c, Normal massula of strain 204. All bars = 50 μm.

sporulation in *Azolla*. However, it is also clear that while a reduction in temperature promoted sporulation in some strains, in others sporulation was inhibited and it is evident from this that sporulation in a particular strain will only occur within a certain temperature range. Similarly, Ashton (1977) found that sporulation in *A. filiculoides* was greatly reduced at temperatures above 27°C or below 22°C. Kannaiyan and Rains (1985) and Palaywal and Paderon (1986) have recently shown that lower temperatures favor sporocarp formation, although to a large extent with different strains from those used here.

Our results suggest that sporulation in a number of strains of *Azolla* can be induced at will by certain temperature regimes. However, under the conditions used, megasporocarps were sometimes few in number or of abnormal structure. Thus conditions were not ideal for megasporocarp development, while allowing (indeed stimulating) the formation of numerous microsporocarps. This observation opens up the possibility that either micro- or megasporocarps could be induced at will, although further work on factors leading to normal development of the latter is essential for breeding programmes. It is possible that factors other than temperature may determine the proportions of mega- to microsporocarps formed in culture.

The formation of additional floats in the megaspore apparatus of *A. pinnata* strains is not unique, having been previously described in scanning electron microscopic studies (Sweet & Hills, 1971) on herbarium collections of *A. pinnata*. However, it is disturbing that features often used in *Azolla* taxonomy (Perkins et al., 1985) are apparently readily modified by inadequate conditions for sporulation. Scanning electron microscopy of strains 202, 203, and 204 of *A. mexicana* displayed differences in megaspore apparatus architecture between and within the three strains of what is thought to be *A. mexicana*, although the identification of these strains is still in some doubt. Although we agree with Fowler and Stennett-Willson (1978) that in the past too much reliance may have been placed on the use of vegetative features for the identification of *Azolla*

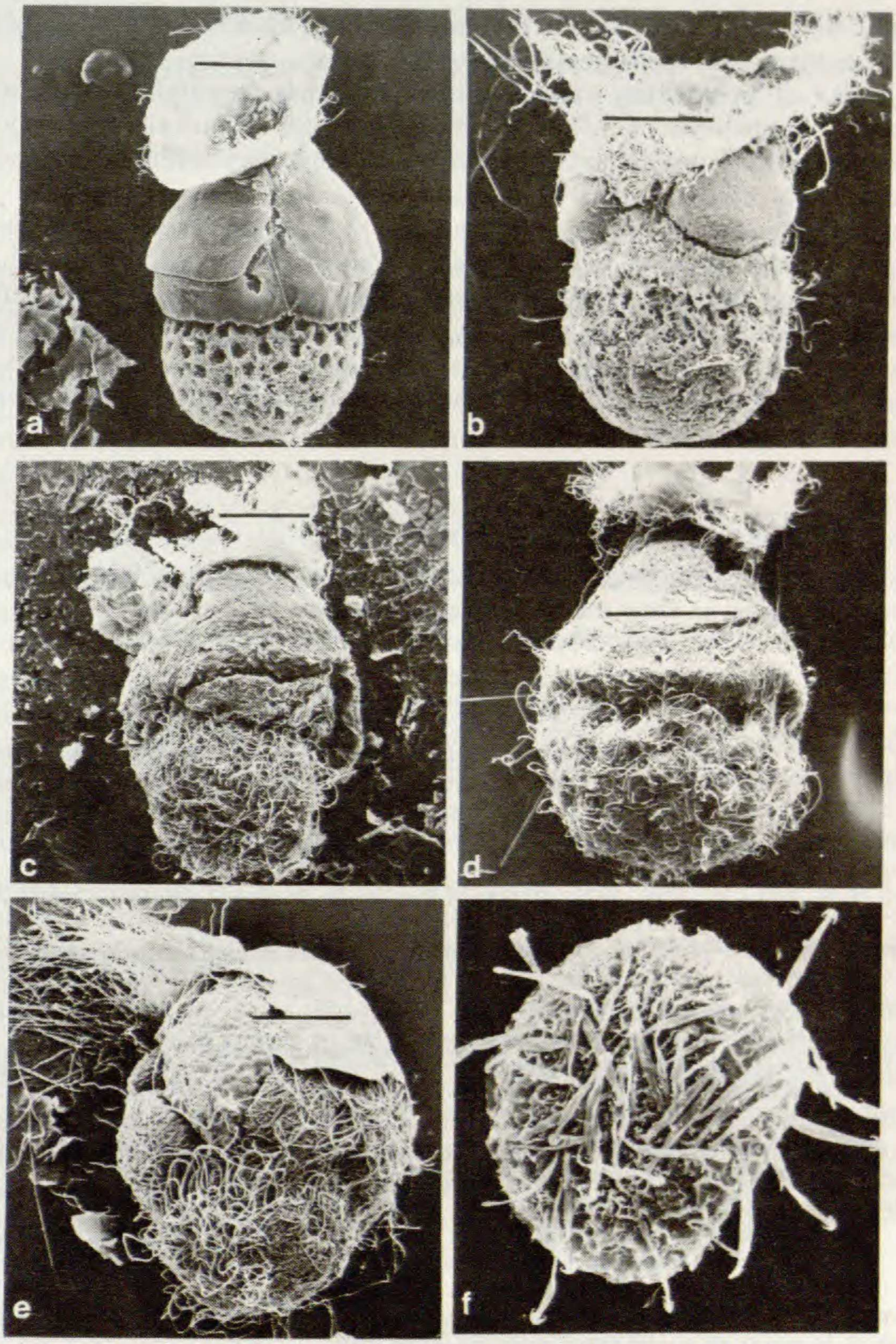

FIG. 3. Megaspore apparatus of *A. mexicana* cultured under various conditions. a, Megaspore apparatus of strain 201 cultured under normal 'control' conditions (26/18°C). b, Abnormal megaspore apparatus of strain 202 from a culture in reduced temperature conditions (day/night: 21/13°C). c, Abnormal megaspore apparatus of strain 203 from a culture under reduced temperature conditions (21/13°C). d, Abnormal megaspore apparatus of strain 204 from a culture under reduced temperature conditions (21/13°C). e, Abnormal megaspore apparatus of strain 204, from a culture under reduced temperature conditions (21/13°C). f, Normal massula from strain 201 cultured under normal 'control' conditions (26/18°C). All bars = 100 μm.

species, we would recommend that taxonomists be wary when using the megaspore apparatus to define a species.

In terms of promoting sporulation the growth regulating substance experiments were a complete failure. Although a range of concentrations known to be effective in other circumstances was used, it remains possible that higher or lower amounts might have had some effect.

In conclusion we suggest that it is unlikely that any one factor alone can be used to promote sporulation in all species and strains of *Azolla*, and that a combination of factors, including a particular temperature range, has to be operative for an individual strain to produce normal mature micro- and megasporocarps in relatively equal proportions. Further work is needed, particularly with a view to elucidating the factors controlling the development of micro- and megasporocarps.

Acknowledgments

The authors thank Jill Howard and Ian Miller for technical and photographic assistance. The work was supported by grant no. R3637 from the Overseas Development Administration.

Literature Cited

Ashton, P. J. 1977. Factors affecting the growth and development of *Azolla filiculoides* Lam. Proc. 2nd Natl. Weeds Conf. South Africa. 249–268, ed. E. G. H. Oliver. Rotterdam: A. A. Balkena.

Fowler, K. and J. Stennett-Willson. 1978. Sporoderm architecture in modern *Azolla*. Fern Gaz. 11:405–412.

Holst, R. W. and J. H. Yopp. 1979. Studies of the *Azolla-Anabaena* symbiosis using *Azolla mexicana*, 1. Growth in nature and laboratory. Amer. Fern J. 69:17–25.

Kannaiyan, S. and D. W. Rains. 1985. Studies on the influence of temperature on sporulation in *Azolla*. Natl. Acad. Sci. Lett. 8: 229–231.

Nickell, L. G. 1961. Physiological studies with *Azolla* under aseptic conditions. II. Nutritional studies and the effects of chemicals on growth. Phyton (Buenos Aires) 17:49–54.

Palaywal, P. C. and E. M. Paderon. 1986. Sporocarp formation and spore germination of *Azolla* strains in the Philippines. Phil. Agri. 69:633–643.

Perkins, S. K., G. A. Peters, T. A. Lumpkin, and H. E. Calvert. 1985. Scanning electron microscopy of the perine architecture as a taxonomic tool in the genus *Azolla* LAMARCK. Scanning Electron Microscopy/1985/IV:1719–1734. Chicago: SEM Inc., AMF O'Hare.

Peters, G. A., R. E. Toia, Jr., W. R. Evans, D. K. Crist, B. C. Mayne, and R. E. Poole. 1980. Characterization and comparisons of five N_2-fixing *Azolla-Anabaena* associations. I. Optimization of growth conditions for biomass increase and N content in a controlled environment. Plant, Cell and Env. 3:261–269.

Sweet, A. and L. V. Hills. 1971. A study of *Azolla pinnata* R. Brown. Amer. Fern J. 61:1–13.

Talley, S. N. and D. W. Rains. 1980. *Azolla filiculoides* Lam. as a fallow season green manure for rice in a temperate climate. Agron. J. 72:11–18.

Tung, H. F. and I. Watanabe. 1983. Differential response of *Azolla-Anabaena* associations to high temperature and minus phosphorus treatments. New Phytol. 93:423–431.

Watanabe, I., Bai Ke-Zhi, N. S. Berga, C. R. Espinas, O. Ito, and B. P. R. Subudhi. 1981. The *Azolla-Anabaena* complex and its use in rice culture. IRRI Research Paper Series 69.

——— and N. S. Berja. 1983. The growth of four species of *Azolla* as affected by temperature. Aquatic Bot. 15:175–185.

———, C. R. Espinas, N. S. Berja, and B. V. Alimagno. 1977. Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. IRRI Research Paper Series 11:3–4.